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Relationship Between Protective Effects of Estrogen, ApoE and Alzheimer's Disease

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Relationship Between Protective Effects of Estrogen, ApoE and Alzheimer's Disease

(TITLE)

BY

Sara M. Ludwig, B.A.

THESIS

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Relationship Between Protective Effects of Estrogen, ApoE and Alzheimer's Disease

A Thesis
Presented To The
Faculty Of
Department Of Biological Sciences
At
Eastern Illinois University

In Partial Fulfillment
Of The Requirements For The Degree
Master of Science
In
Biological Sciences

By
Sara M. Ludwig, B.A.
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Abstract

Alzheimer's disease (AD) is a neurodegerative disorder characterized by progressive memory loss and loss of cognitive function. The pathogenesis and progression of AD is poorly understood. Recently, several risk factors have been determined, however, how these risk factors function to induce AD onset has yet to be elucidated.

Apolipoprotein E genotype has been clearly demonstrated to be a risk factor for AD. The apoE2 and apoE3 isoforms appear to be protective against AD, whereas the apoE4 isoform has been implicated in the development of AD. The apoE4 allele works in a dosage-dependent fashion; that is, the greater the expression of apoE4, the earlier the onset of AD and the quicker the progression. ApoE has been clearly shown to play a central role in nerve repair and regeneration in the central nervous system (CNS) and peripheral nervous system (PNS), however, its precise functions in these repair processes remain unclear.

Recently, it has been acknowledged that significantly more females are afflicted with AD than males; this has been suggested to be due to estrogen loss at menopause. Several studies have shown that women receiving estrogen replacement therapy (ERT) have a reduced risk of developing AD, and if they do develop AD, the progression is slower and the age of onset is later. It is thought that estrogen somehow modulates apoE levels and/or function in the CNS, however, estrogen's role in the CNS is poorly understood.

The major objective of this study was to determine the effects of estrogen on nerve regeneration in the olfactory system of mice. In order to study the effects of

estrogen on nerve regeneration, mice were ovariectomized (OVX) and divided into four treatment groups. Estrogen or placebo pellets were implanted in the nape of the animals' necks and mice were then irrigated with either Triton (lesioned) or saline (control). Western immunoblotting techniques were then used to quantitate specific marker proteins in the olfactory bulbs. These biochemical markers shed light on regeneration events occurring within the system.

Results indicated that estrogen does indeed play a significant role in CNS repair. Lesioning was shown to recruit glial cells to the site of injury (shown by blotting for GFAP). These glial cells then up-regulate apoE in order to facilitate neuronal repair, as shown by apoE immunoblots. Estrogen treated lesioned animals showed significantly more nerve repair (shown by olfactory marker protein or OMP blots) than in the lesioned mice receiving no estrogen. Finally, blots for synaptophysin indicated that more synapses were formed in the estrogen treated lesioned group as compared to the lesioned group not receiving estrogen. In all cases, there were no significant differences between the two non-lesioned groups.

These results strongly support the notion that estrogen facilitates apoE function in nerve repair in the CNS. Further studies at the molecular level are required to understand how estrogen and apoE work in concert to facilitate nerve repair.

Introduction

Apolipoprotein E (apoE) is a 34-kDa-protein component of very low-density lipoproteins (VLDL), chylomicrons, chylomicron remnants, and high-density lipoproteins (HDL) (Mahley, 1988). In its mature form in plasma and cerebrospinal fluid (CSF), apoE is 299 amino acids in length (Mahley, 1988). ApoE plays a central role in the regulation of lipoprotein metabolism and in the control of lipid transport and lipid redistribution among target tissues and cells (Weisgraber, 1994). Lipid transport and redistribution is regulated by apoE via interaction with lipoprotein receptors (Mahley, 1988). Receptor-lipoprotein binding initiates cellular uptake and degradation of the lipoproteins. The lipid becomes available for utilization in the regulation of intracellular cholesterol metabolism. ApoE, therefore, serves as a ligand for the receptor-mediated clearance of lipoproteins from the plasma (Rall et al., 1982).

The apoE gene is 3597 nucleotides in length and contains four exons (Mahley, 1988). ApoE is encoded by an 1163-nucleotide mRNA (Mahley, 1988). In humans, there are three major isoforms of apoE, designated as apoE2, apoE3, and apoE4, that are products of the three alleles (designated ε2, ε3, and ε4) located at a single gene locus on the long proximal arm of chromosome 19 (Mahley, 1988). The molecular basis of this polymorphism of the apoE gene results from cysteine-arginine interchanges at two positions in the apoE protein (Weisgraber, 1994). These single amino acid substitutions are found at residues 112 and 158 (Rall et al., 1982). The most common isoform, apoE3, contains cysteine at residue 112 and arginine at position 158. ApoE2 has cysteine at both positions and apoE4 contains arginine at both positions (Weisgraber, 1994).

Many organs synthesize apoE; the largest quantity is made in the liver, followed by the brain (Boyles et al., 1985). In these organs, a wide variety of cell types are capable of producing apoE, which includes macrophages (Mahley, 1988), astrocytes (Pitas et al., 1987) and oligodendrocytes (Stoll et al., 1989). In addition to its function in lipid metabolism as previously mentioned, apoE has also been implicated in a wide variety of other physiological processes throughout the body. ApoE may have a structural role in a number of lipoprotein particles as well as regulating their metabolism (Wisniewski and Frangione, 1992). ApoE is also thought to function in immunoregulation (Cuthbert and Lipsky, 1984), nerve repair and regeneration in both the central nervous system (CNS) and the peripheral nervous system (PNS), nerve growth (Ignatius et al., 1986; Ignatius et al., 1987; Snipes et al., 1986; Boyles et al. 1989; Handelmann et al. 1992), modulation of intracellular cholesterol utilization (Reyland et al., 1991), steroidogenesis in adrenal cells (Reyland and Williams, 1991), and as an activator or modulator of hepatic lipase (Ehnholm et al., 1984; Landis et al., 1987; Thurin et al., 1991, 1992). ApoE is thought to participate in the mobilization and redistribution of lipids during normal development of the nervous system (Pitas et al., 1987) and in the regeneration of peripheral nerves after injury (Boyles et al., 1989).

The exact function of apoE in the nervous system is poorly understood, but the mechanisms in which it is involved are of particular interest. ApoE levels increase in response to injury in both the CNS and the PNS (Ignatius et al., 1986). In rats, the synthesis of apoE increases by 250- to 350-fold within three weeks following peripheral nerve injury (Snipes et al., 1986; Boyles et al., 1989). It has also been reported that macrophages synthesize and release apoE following peripheral lesion which accumulates

to 5% of total extracellular protein (Skene and Shooter, 1983). It has been suggested that this accumulation of apoE is to scavenge cholesterol from the degenerating myelin and recycle it to the growth cones of sprouting axons by LDL-receptor-mediated endocytosis for membrane biosynthesis (Mahley, 1988; Goodrum et al., 1995; Poirier et al., 1993).

Based on these observations, it has been proposed that apoE is involved in neurodegenerative processes by isoform-specific effects on neurite outgrowth and cytoskeletal stability (Mahley et al., 1995; Weisgraber and Mahley, 1996). In vitro studies have shown that addition of apoE3 to dorsal root ganglion neurons in culture stimulated neurite outgrowth whereas apoE4 decreased neurite extension (Nathan et al., 1994). These data imply that apoE is important for peripheral nerve regeneration (Mahley, 1988). The data from apoE knock out (apoE KO) mice, however, does not support this hypothesis. Regenerating nerves in both control mice and apoE KO mice were morphologically identical at two and four weeks following sciatic nerve crush (Popko et al., 1993; Goodrum, 1995). This suggests that other apolipoproteins in the PNS may substitute for apoE when it is absent. Hence, the specific role of apoE and its importance in the PNS remains unclear.

The precise function and mechanism of action of apoE is even less clear in the central nervous system. ApoE is the principle apolipoprotein in the brain and cerebrospinal fluid (CSF). The majority of apoE in the CNS is synthesized and secreted primarily by glial cells, and by microglia to a lesser extent (Pitas et al., 1987; Pitas et al., 1987; Borghini et al., 1995; Boyles et al., 1985; Naikai et al., 1996). ApoE is the only apolipoprotein in the CNS that is able to interact with lipoprotein receptors (Pitas et al., 1987; Borghini et al., 1995). Cells within the brain express four receptors for apoE-

containing lipoproteins: the low density lipoprotein (LDL) receptor, the LDL receptor-related protein (LRP), the very low density lipoprotein (VLDL) receptor, and the glycoprotein (gp) 330. The LDL receptor and the LRP are expressed by neurons (Pitas et al., 1987; Boyles et al., 1985). It has been shown that the VLDL receptor in the CNS is expressed in some human neurons, whereas gp330 is expressed by brain ependymal cells (Willnow et al., 1992; Sakai et al., 1994; Kim et al., 1996; Kounnas et al., 1994). It has been reported that human apoE-containing lipoproteins bind to fibroblast LDL receptors and that the LDL receptor and the LRP mediate the binding and internalization of apoE-containing lipoproteins in cultured neurons (Bellosta et al., 1995). These studies provide evidence that the apoE and apoE-containing lipoproteins are present within the brain where they can interact with neurons and that lipoprotein transport by apoE is important for normal functioning of adult neurons.

Increased apoE immunoreactivity is present in the brains of patients with such neurological disorders as Alzheimer's disease (AD), Down's syndrome, and Creutzfeld-Jacob disease (Namba et al., 1991). It has been demonstrated that expression of apoE increases following optic nerve injury, but absolute levels of apoE do not increase (Ignatius et al., 1986). ApoE mRNA is increased in the brains of AD patients (Diedrich et al., 1991) and in response to injury in both the PNS (Boyles et al., 1989) and CNS (Snipes et al., 1986).

It has been shown that addition of apoE3 to a culture stimulates neurite outgrowth in transformed murine neuroblastoma (Neuro-2a) cells, whereas apoE4 inhibits neurite extension (Bellosta et al., 1995). Recent studies show that apoE knock out (apoE KO) mice display significant synaptic loss and disruption of the dendritic cytoskeleton with

age and a reduced recovery following perforant pathway lesioning (Masliah et al., 1995; Masliah et al., 1996; Masliah et al., 1997). Synaptophysin (a marker presynaptic terminals) and microtubule-associated protein (MAP-2, a dendritic marker) levels in the hippocampus and neocortex of apoE KO mice were shown to decrease as compared to age-matched control mice. However, other studies have not observed any significant morphological deficits in apoE KO mice (Anderson et al., 1998). The reasons underlying these inconsistencies are not clear, but differences in the strain and age of both the apoE KO and the control mice used may have contributed to the inconsistent results observed.

In contrast to the morphological studies, behavioral studies have consistently shown that apoE KO animals exhibit spatial learning deficits (Masliah et al., 1996; Gordon et al., 1995; Gordon et al., 1996). Infusion of recombinant apoE into the lateral ventricles of apoE KO mice reversed behavioral and morphological anomalies (Masliah et al., 1996). Other studies involving apoE KO mice have suggested that apoE may be involved in protecting the brain against acute injury (Chen et al., 1997). These results provide convincing evidence that apoE plays a critical role in neuroprotection, preservation, and plasticity within the CNS.

Studies of apoE within the CNS have shed some light on neurodegenerative disorders, especially Alzheimer's disease (AD). ApoE genotype has been shown to be a major risk factor for AD. ApoE immunoreactivity is associated with neurofibrillary tangles and neuritic plaques, the characteristic pathological structures present in the brains of AD patients (Namba et al., 1991; Wisniewski and Frangione, 1992; Schmechel et al., 1993; Strittmatter et al., 1993). The neuritic plaques are, for the most part, extracellular and constitute classical amyloid deposits and often a neuritic component.

The major protein present in these plaques is the amyloid beta peptide (Ab), which is formed by cleavage of the amyloid precursor protein (APP) (Haass and Selkoe, 1993). It has been demonstrated that apoE is associated with Ab deposits in neuritic plaques and in the angiopathy of cerebral vessels (Strittmatter et al., 1993). Unlike neuritic plaques, neurofibrillary tangles are intracellular and contain structures known as paired helical filaments (Goedert et al., 1992). The role of these plaques and tangles in the progression of AD is not yet clear.

There are three forms of AD: early-onset familial, late-onset familial, and late-onset sporadic. Early-onset AD represents approximately 5% of patients, whereas late-onset AD accounts for a majority of AD cases. Recent studies have indicated a relationship between the apoE4 allele and late-onset familial AD (Strittmatter et al., 1993) as well as late-onset sporadic AD (Tsai et al., 1994). It has been demonstrated that the risk of early-onset AD and disease progression increase is related to the number of apoE4 alleles in a dose-dependent fashion (Tsai et al., 1994; Corder et al., 1993). The frequency of the apoE4 allele has been shown to be greatly over-represented in late-onset familial AD patients (representing 52% of the subjects) versus controls (16%), and the risk of AD in individuals homozygous for the apoE4 allele is over five times that of homozygous apoE3 individuals (Corder et al., 1993).

Another recent study suggests that the apoE4 allele may also be involved with age of onset in Parkinson's disease. This evidence strongly suggests that there is a definite correlation between apoE polymorphism and the development of neurodegenerative disorder (Nisar, 1999). The mechanism behind the pathogenesis of these disorders and the exact effects of apoE on CNS neurons remain unclear. One possible mechanism of

apoE in AD may involve neuronal plasticity, based on previous studies which suggest that apoE may play a crucial role in nerve regeneration.

In addition to apoE genotype, it seems that estrogen plays an important protective role in the development of AD. The risk of AD and related dementia for women who used estrogen replacement therapy (ERT) was reduced by about one third below that of women who had never used ERT (Paganini-Hill and Henderson, 1996). Recent studies have suggested that estrogen's protective effects are through its action as a trophic factor for cholinergic neurons, a modulator for the expression of apoE in the brain, an antioxidant compound decreasing the neuronal damage caused by oxidative stress, and a promoter of the physiological nonamyloidogenic processing of the APP, decreasing the production of the Ab protein (Inestrosa et al., 1998).

It has been demonstrated that ERT increases cerebral and cerebellar blood flows in postmenopausal women (Ohkura et al., 1995). Cerebral blood flow values are shown to be higher in females than in males until the age of 50 (Davis et al., 1983) or 60 (Shaw et al., 1984). Furthermore, ERT improves cognitive functions and increases regional cerebral blood flow in female patients with dementia of the Alzheimer type (Ohkura et al., 1994).

Estrogen receptors have been reported in astroglia, sheathing glia, and microglia (Azcoitia et al., 1999; Mor et al., 1999). This suggests that estrogen may be able to "activate" glial cells (Struble, personal communication). However, a simple activation or inactivation of glial cells by estrogen has not been reported *in vivo*. One study demonstrated that estrogen effects on glia represented an interaction between neurons and glial cells (Stone et al., 1998). Estrogen appears to regulate apoE gene expression in an

organ specific manner (Srivastava et al., 1996). A 1.4-fold increase in apoE mRNA in the brain followed after five days of 17β-estradiol administration in mice (Struble, personal communication). However, several other organs (including the liver and intestine) displayed no changes.

It has been clearly shown that estrogen replacement therapy (ERT) reduces the risk of AD and also delays the age of clinical onset (Waring et al., 1999; Paganini-Hill, 1994). It has also been found that estrogen replacement in experimental animals promotes recovery from neurological damage (Stone et al., 1998; Stone et al., 1997; Toung et al., 1998), and that estrogen administration increases apoE and glial markers. The exact mechanism by which estrogen facilitates nerve repair is still unknown.

Recent studies have shown that ERT has no detectable effect on cognitive functions in neurologically intact postmenopausal women. Parallel studies in mice, evaluating the effects of long-term estrogen elevation, found only transient changes in the synaptic density of olfactory neurons (Nathan, personal communication). These results suggest that estrogen's effects on the CNS, in neurologically intact animals, are only transitory.

Based on these previous studies I hypothesized that 1) estrogen's main effect in the CNS is to facilitate the repair process, and will only have a mild and transitory effect in the absence of damage; and 2) estrogen facilitates repair by up-regulating apoE.

The major aim of this study is to determine the effects of estrogen on apoE level, glial cells, and synaptic density in the olfactory bulb of control mice following olfactory nerve lesioning. The olfactory system is selected as a model because lesioning techniques may be used to amplify tissue repair processes that normally occur in this system.

Western immunoblotting will be used to determine differences in apoE, synaptophysin, GFAP, and OMP levels in lesioned and non-lesioned mice. All mice will be ovariectomized (OVS) in order to study the effects of estrogen and the absence of estrogen on these marker proteins.

Materials and Methods

Animals

Breeding pairs of C57BL6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Two to three month old female mice were used.

Treatment Groups

Animals were randomly divided into 4 treatment groups (n = 5-8 for each group) as follows:

- ovariectomized /Estrogen pellet/Triton irrigated (lesioned)
- ovariectomized /Placebo pellet /Triton irrigated (lesioned)
- ovariectomized /Estrogen pellet /Saline irrigated (control)
- ovariectomized /Placebo pellet /Saline irrigated (control)

Surgery

Animals were either ovariectomized (OVX) or sham operated, depending on the treatment group. All animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (80 mg/kg). The incision site was shaved and an aseptic bilateral dorsal incision made just under the rib cage. The fallopian tube was clamped just below the ovary and the ovary was then completely removed. The muscle layer was sewn with silk ligatures and the skin was closed with autoclips. The same procedure was followed on the opposite side.

Pellet Implantation

A 17-β estradiol pellet (SE-121, Innovative Research of America, Sarasota, Florida) or a placebo pellet (SC-111, Innovative Research of America, Sarasota Florida) was implanted into each animal in the appropriate treatment groups at the time of surgery. The pellet was placed just under the skin at the back of the animals' necks with a 12 Gauge trochar.

Nasal Irrigation

Three days after surgery and pellet implantation, mice were irrigated with 0.9% saline (SL) or lesioned with 0.7% Triton X-100 (TX), as previously described (Verhaagen et al., 1990) depending on treatment group. Briefly, a 25 gauge needle 10 mm in length with a rounded tip, was inserted about 2 mm into one nostril, and 100 μL of 0.7% Triton X-100 (BP151-500, Fisher) in 0.9% saline or 0.9% saline (control) was squirted into a nostril of unanesthetized mice (Verhaagen et al., 1990). The excess solution was drained from the nasal passage by gently shaking the mice. This technique results in complete bilateral nerve lesion (Verhaagen et al., 1990).

Sacrifice

Mice were sacrificed 17 days after surgery and pellet implantation. Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (80mg/kg). After the animals were deeply anesthetized, a needle was inserted transcardially and the animals were thoroughly perfused with phosphate buffered saline (pH 7.4). The olfactory bulbs were dissected and processed as described below.

Tissue Preparation

The olfactory bulbs were homogenized in ice cold TMN buffer (25 mM Tris-HCl [pH 7.6] 3 mM $MgCl_2$, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) (Xu et al., 1996). The homogenate was lysed by adding 1% Triton X-100, 0.5% deoxycholate, and 0.2% SDS on ice for 5 min (Xu et al., 1998). The homogenate was then centrifuged for 2 min in a microcentrifuge (g = 13,000) (Xu et al., 1998). The supernatant was saved for protein assay and western immunoblot analysis.

Protein Assay

Protein assay was performed by Lowry protein assay method modified by Peterson (Peterson, 1977). Briefly, 5 µl of homogenized olfactory bulb sample was diluted to 50 µl with double distilled water, and10 µl of diluted samples (in triplicates) were protein assayed. The volume of each of the 10 µl triplicates was brought to 400 µl with double distilled water. Four hundred microliters of Lowry Reagent A (equal volume of dH_2O, 0.8N NaOH, copper tartarate carbonate, and 10% SDS) was added and the samples vortexed. Following 10 min of incubation at room temperature, 200 µl of Lowry Reagent B (1 part Folin-Ciocalteu's phenol reagent [F-9252, Sigma, St. Louis, MO] and 5 parts of dH_2O was added, vortexed, and incubated for 50 min at room temperature for color development. Absorbance was recorded at 750 nm using a spectrophotometer. Bovine albumin serum (A-7511, Sigma, St. Louis, MO) was used as a standard sample for the protein assay.

SDS – Polyacrylamide Gel Electrophoresis

Proteins in the olfactory bulb were resolved by SDS-PAGE as previously described (Bellosta et al., 1995). Briefly, 20 μg of olfactory bulb protein was mixed with an equal volume of 2X Lammeli sample buffer (6.25 ml 4X Tris/SDS [pH 6.8], 5 ml glycerol, 1g SDS, 0.5 ml 2-mercaptoethanol, bromophenol, 13.25 ml dH_2O). Samples were boiled for 5 min and then centrifuged at 14,000 g for 5 min. The gel cassettes were inserted into the buffer tank of an EC120 Mini gel vertical system (E-C Apparatus Corporation, St. Petersburg, FL) containing 1X running buffer, pH 8.3 [250 ml of 5X running buffer (15 g Tris-base, 72 g glycine, 5 g SDS, 750 ml dH_2O].

The samples and 5 μl of kaleidoscope prestained standards (161-0324, Bio-Rad Laboratories, Hercules, CA) were electrophoresed through a pre-cast 4-20% gradient gel (Fisher, FB3435). Samples were electrophoresed at 80 volts until separation began, and then at 140 volts until the dye front reached the bottom of the gel.

Protein Transfer

Following electrophoresis, the gel was placed in transfer buffer (3.03 g Tris-base, 14.4 g glycine, 200 ml methanol, 800 ml dH_2O) on a shaker. The transfer membrane (Immobilon-P IPVH00010, Millipore, Bedford, MA) was soaked in methanol for 5 sec and then washed in dH_2O for 5 min. The gel was placed on presoaked filter paper in the holder and the transfer membrane was placed on top of the gel. Using a trans-blot transfer cell (170-3930, Bio-Rad), proteins from the gel were transferred onto the membrane by passing 100 volts for an hour.

Western Immunobloting

ApoE

ApoE was quantified as previously described (Xu et al., 1996). Briefly, the blots were incubated in polyclonal goat antiserum against human apoE (178479, Calbiochem, San Diego, CA) (1:5,000 dilution in T-TBS [pH 7.6] 0.1M Tris, 0.15M NaCl, 0.1% Tween-20) for 30 min on a shaker at room temperature. The membrane was then washed 4 times (10 min each) in T-TBS. The blot was then incubated in the secondary antibody solution (rabbit anti-goat IgG-HRP [AP106P, Chemicon, Temecula, CA] 1:10,000 dilution) for 30 min on a shaker at room temperature. Blots were washed with T-TBS 5 times (10 min each) (Xu et al., 1996). Immunoreactive bands were then visualized with SuperSignal West Pico Chemiluminescent substrate (34080, Pierce, Rockford, IL) and then exposed to BioMax film (Kodak).

GFAP

To quantitate GFAP, blots were incubated in mouse anti-GFAP (BYA60771, Accurate Chemical & Scientific Corp, Westbury, NY) (1:2,000 dilution in TBST) for on hour on an orbital shaker at room temperature. Blots were then washed 4 times (10 min each) in TBST, pH 7.5 [20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 0.1 g BSA]. Blots were incubated in secondary antibody solution (goat anti-mouse IgG-HRP) [AP124P, Chemicon, Temecula, CA] 1:1,000 dilution in TBST) on an oribital shaker for one hour at room temperature. The blots were then washed 5 times (10 min each) in TBST. Visualization of the bands was done using the same protocol as previously described for apoE.

OMP

The same protocol was used as above, except dilutions differ. Primary antibody solution consists of goat anti-rat OMP (a generous gift from Dr. Frank Margolis, Univ. of Mass) (1:10,000 dilution in TBST). Secondary antibody solution is rabbit anti-goat IgG-HRP (AP106P, Chemicon, Temecula, CA) (1:5,000 dilution in TBST).

Synaptophysin

The same protocol was used as above to quantitate synaptophysin. Primary antibody solution consists of rabbit anti-human synaptophysin (A0010, DAKO, Carpinteria, CA) at 1:2,000 dilution in TBST. The secondary antibody was goat anti-rabbit IgG-HRP (AP132P, Chemicon, Temecula, CA) (1:1,000 dilution in TBST).

Quantitation/Data Analysis

All experiments were repeated at least three times to assure reproducibility of the results. Bands were quantified by densitometry (Scion Image). As an internal control, the blots also contained an olfactory bulb extract from unlesioned animals. A one-way analysis of variance was used to compare treatment means. If a significant F value was found, a Duncan's Multiple Range mean comparison test was used to differentiate significance between and among means.

Results

In this study, mice were ovariectomized (OVX) and either a 17β-estradiol pellet or a placebo pellet was implanted subcutaneously in the nape of the neck. Olfactory nerve lesioning was performed by intranasal irrigation with Triton X-100 (TX) in saline three days after surgery and pellet implantation. The animals were divided into four treatment groups: 1) OVX/lesion/estrogen (TX+E2) (n=5-7), 2) OVX/lesion/placebo (TX+PL) (n=5-7), 3) OVX/no lesion/estrogen (SL+E2) (n=5-7), and 4) OVX/no lesion/no estrogen (SL+PL) (n=5-7). Olfactory bulbs were collected 14 days after lesioning. The expression of four marker proteins: apoE, GFAP, OMP, and synaptophysin were examined using western immunoblotting.

ApoE

Western immunoblotting of apoE revealed a significant increase in the amount of apoE in mice that were treated with estrogen following lesioning of the olfactory nerve (Figure 1). Quantification by densitometry confirmed this with apoE showing significantly higher levels of apoE in the mice with olfactory nerve lesions and estrogen (Figure 2). There were no significant differences in apoE levels in the other experimental groups.

Statistical analysis showed that Bulb levels of apoE in the four treatment groups were not equal ($F_{cal} = 4.31$, $P = 0.017$). ApoE in TX+E2 group were significantly higher than that in the other three groups which were statistically indistinguishable from each other ($P > 0.05$).

GFAP

Western immunoblotting of GFAP demonstrated a significant increase in the amount of GFAP in nerve-lesioned mice, independent of estrogen administration (Figure 3). Quantification by densitometry (Figure 4) confirmed this, showing significantly higher levels of GFAP both of the lesioned groups. The other experimental groups showed no significant differences in expression of GFAP.

Statistical analysis showed that GFAP levels differed among the four treatment groups. Bulb GFAP levels inTX+E2 and TX+PL were significantly ($F_{cal} = 5.86$, $P = 0.0067$) higher than the SL+E2 and SL+PL groups. There were no significant differences ($P > 0.05$) between TX+E2 and TX+PL. Also, there were no significant differences ($P > 0.05$) between the SL+E2 and SL+PL groups.

OMP

Western immunoblotting revealed that OMP levels in the olfactory bulb of TX+E2 were more than 2 times higher than the TX+PL group, although none of the treatment means differed significantly ($F_{cal} = 2.03$, $P = 0.15$). The OMP levels in the TX+E2 group were about 40% of those in the SL+E2 and SL+PL groups. The differences in OMP levels in the SL+E2 group and the SL+PL group were not significant. These results are illustrated in Figure 5.

In sum, OMP expression is increased in the lesioned and estrogen replaced group as compared to the unlesioned and estrogen replaced group (Figure 6), but this trend was not statistically significant, due to variation within the treatment groups.

Synaptophysin

Western immunoblotting of synaptophysin revealed a significant increase in the amount of synaptophysin in the olfactory nerve lesioned and estrogen replaced group, as compared to the lesioned and estrogen deficient group as well as the two unlesioned groups (Figure 7). Quantification by densitometry confirmed this as shown in Figure 8.

Statistical analysis demonstrated that levels of synaptophysin in the olfactory bulbs differed significantly among the four treatment groups. The TX+E2 group was significantly higher ($F_{cal} = 6.13$, $P = 0.0056$) than that in the other three groups, but there was no significant difference between TX+PL, SL+E2, and SL+PL.

Figure 1. Western immunoblot showing 35 kDa apoE protein bands two weeks post-lesioning. Four groups of mice either had their olfactory nerves lesioned using Triton X-100 (+) or exposed to saline (-) and an estrogen pellet (+) or placebo (-) implanted. Results show an increase in the amount of apoE in mice implanted with estrogen pellets that h ad olfactory nerves lesioned (+/+). The relative amounts of apoE in the other groups remained very similar to the controls (-/-).

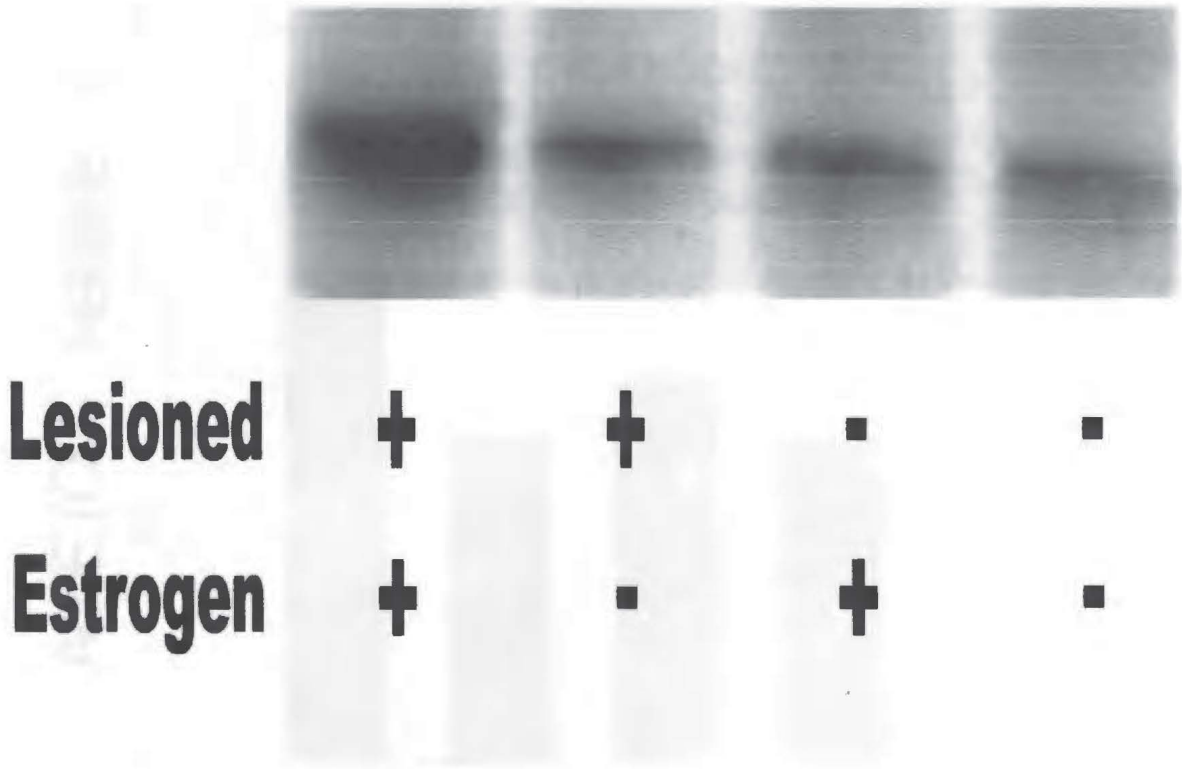


Figure 2. Quantification of apoE in the olfactory bulbs of the same four treatment groups two weeks post-lesion. The lesioned and estrogen replaced group showed significantly higher levels of apoE as compared to the other experimental groups. There were no significant differences in apoE levels for the other three experimental groups.

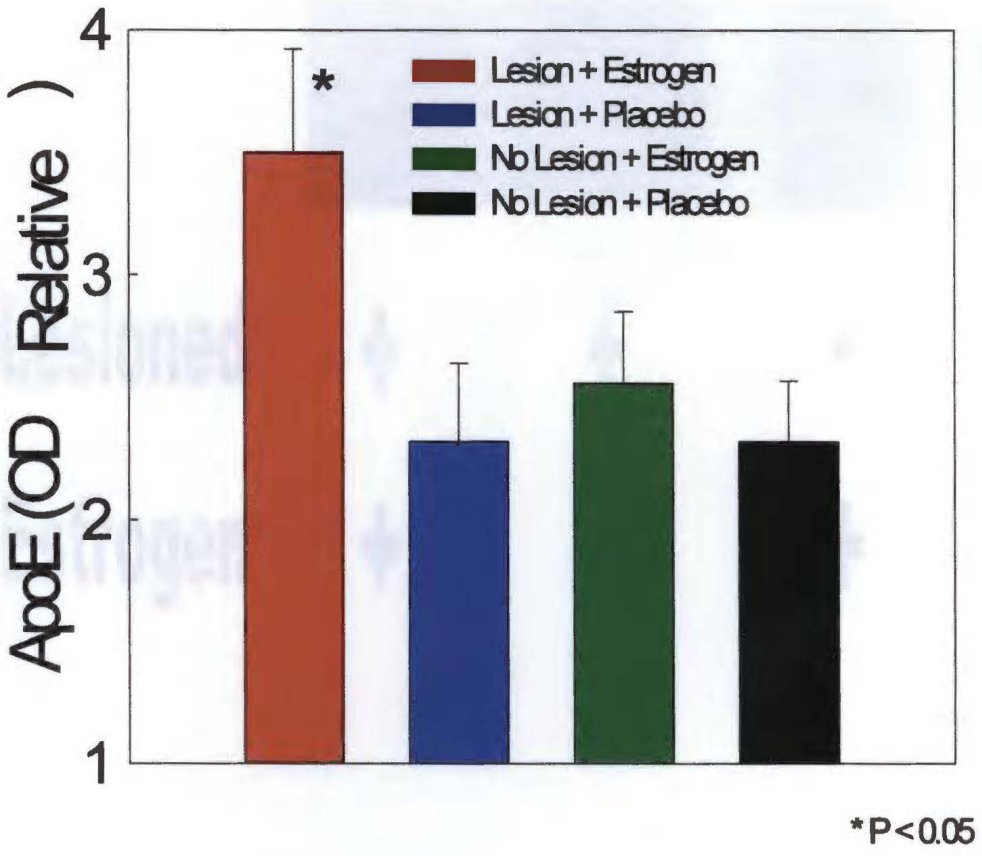


Figure 3. Western immunoblot for GFAP of the same four treatment groups two weeks post-lesion. Results show an increase in the two lesioned groups that is independent of estrogen administration. There are no observable differences between the two unlesioned groups.

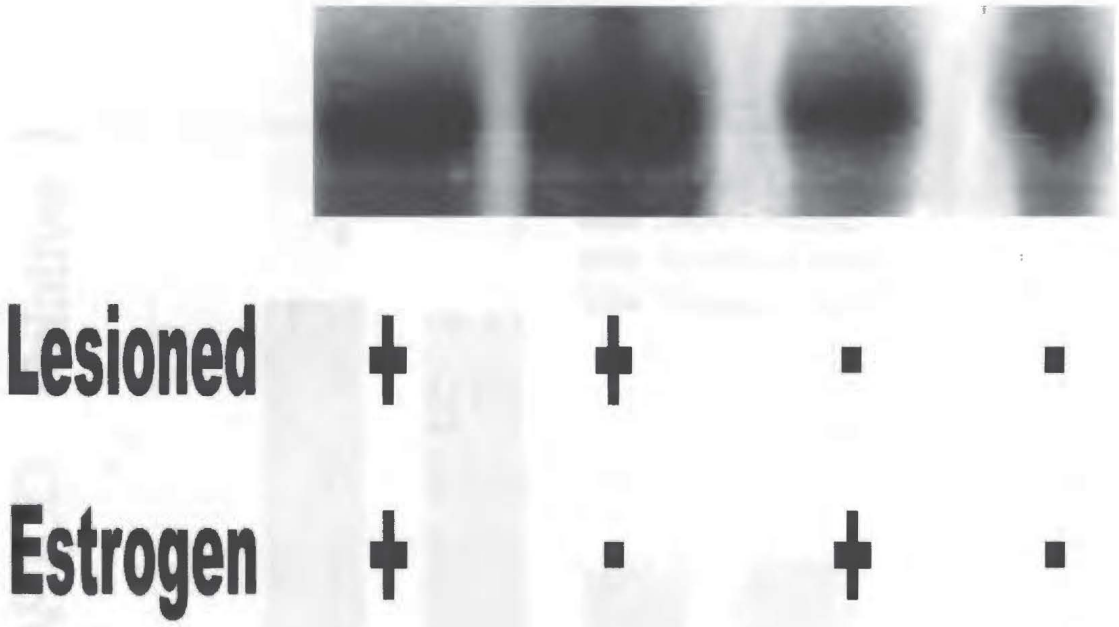


Figure 4. Quantification of GFAP in the olfactory bulb for the same four treatment groups two weeks post-lesion. The two lesioned groups show significantly higher olfactory bulb levels of GFAP than the two unlesioned groups. This increase is independent of estrogen administration. There are no observable differences between GFAP levels in the two lesioned groups and there are no observable differences between the GFAP levels in the two unlesioned groups.

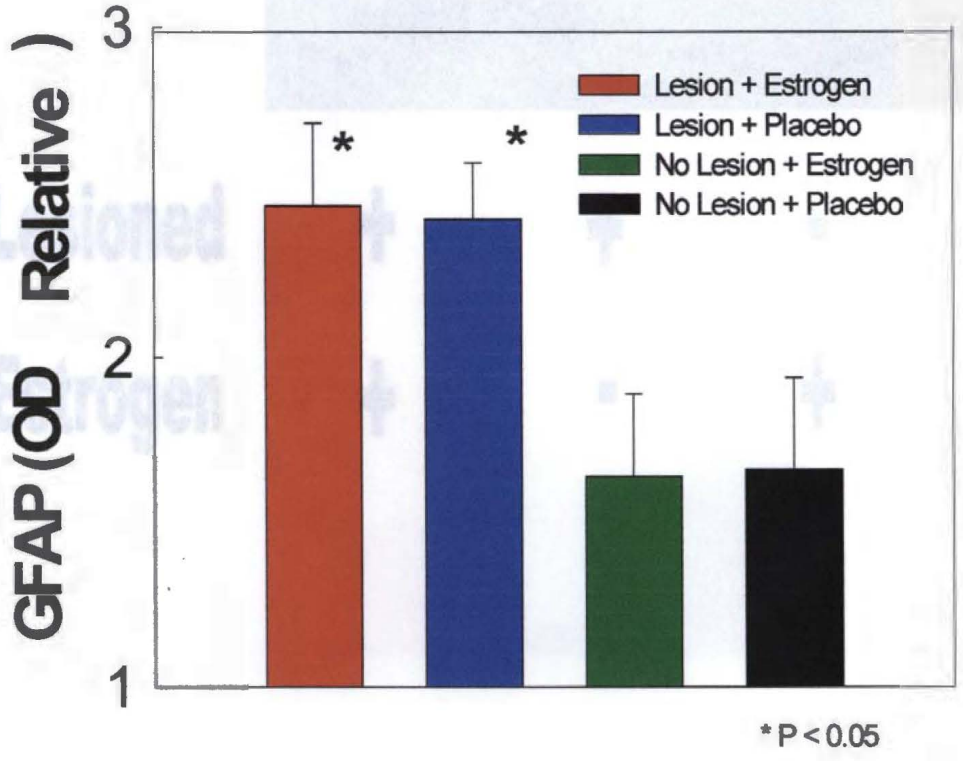


Figure 5. Western immunoblot for OMP for the same four experimental groups two weeks post-lesion. OMP levels in the lesioned and estrogen deficient group are dramatically lower than any of the other experimental groups.

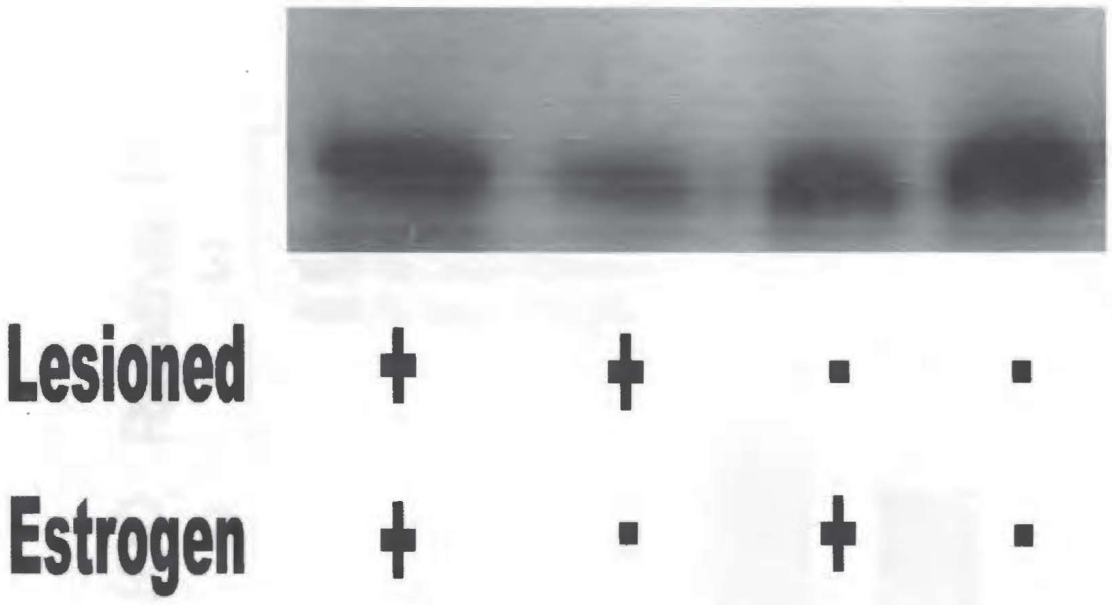


Figure 6. Quantification of OMP in the olfactory bulb for the same four experimental groups two weeks post-lesion. OMP levels in the lesioned and estrogen deficient group were dramatically lower than any of the other experimental groups, including the lesioned and estrogen replaced group. However, these differences were not statistically significant.

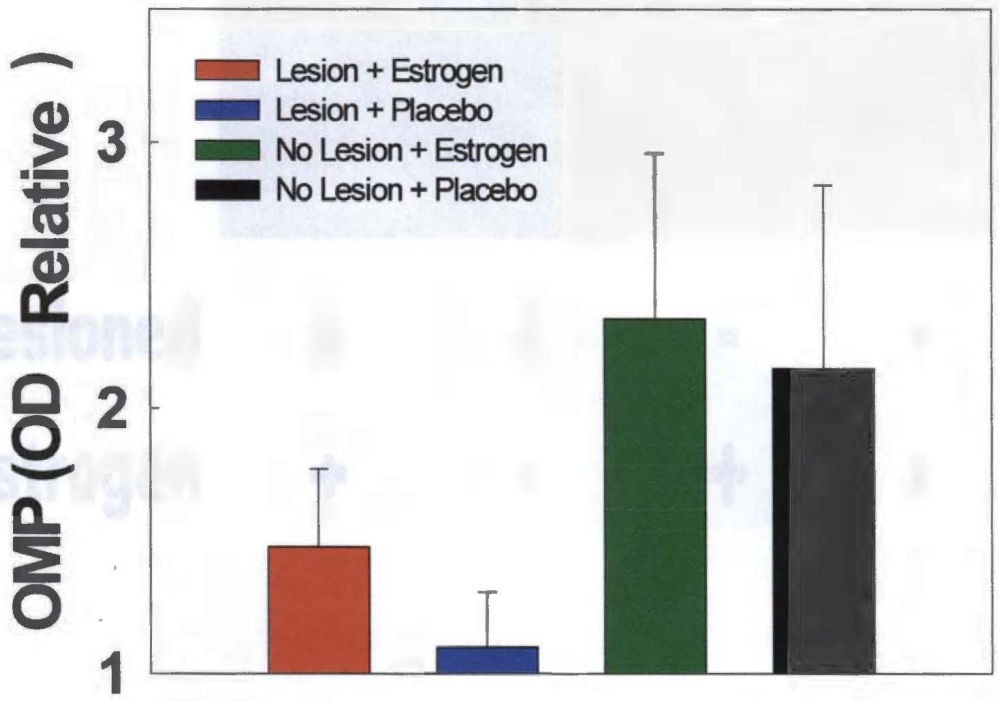


Figure 7. Western immunoblot for synaptophysin levels in the olfactory bulb for the same four experimental groups two weeks post-lesion. Synaptophysin levels were dramatically higher in the lesioned and estrogen replaced group than in any of the other experimental groups. Synaptophysin levels were lowest in the lesioned and estrogen deficient group. There are no observable differences between the two unlesioned groups.

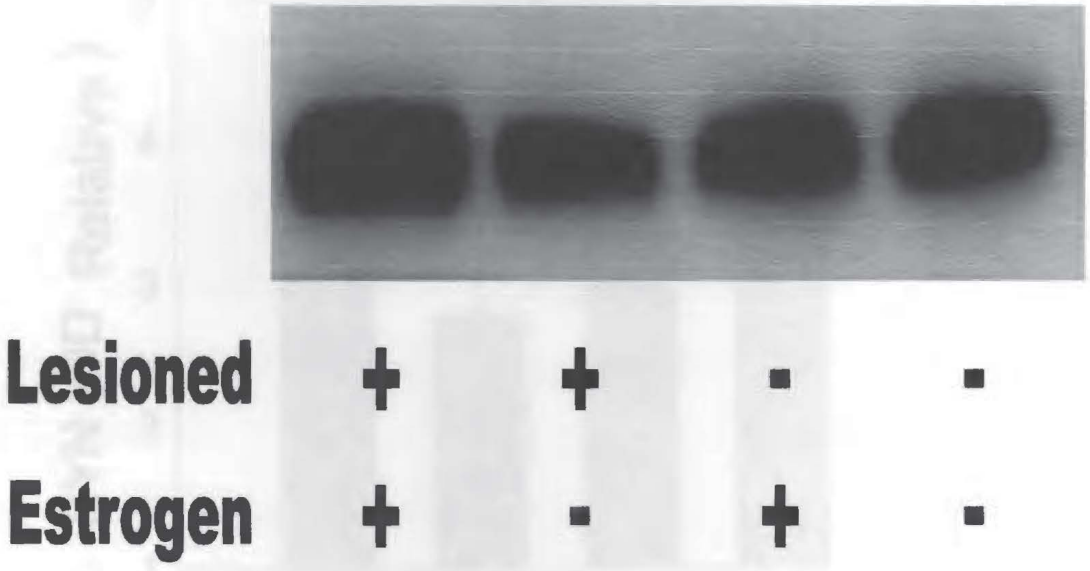
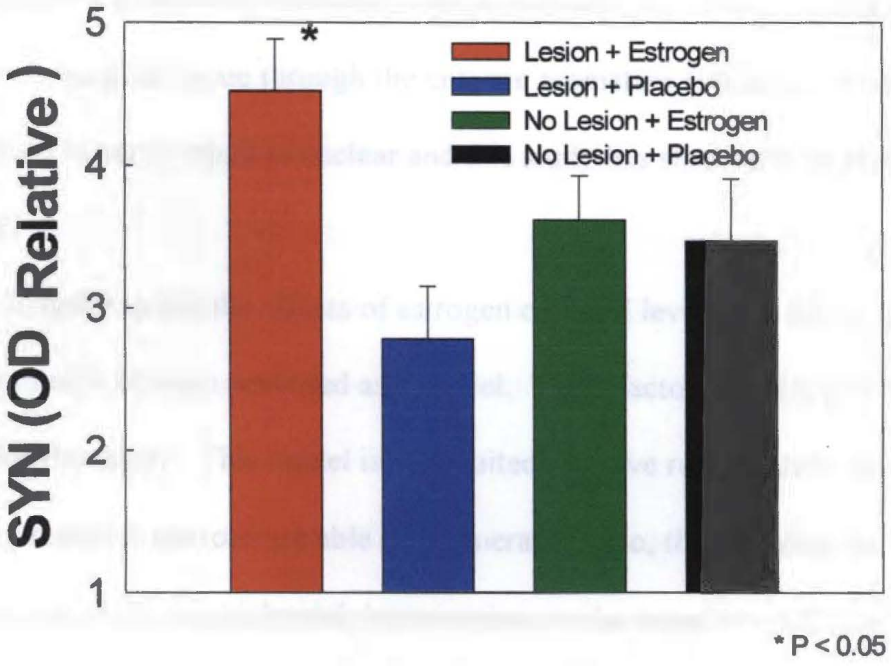


Figure 8. Quantification of synaptophysin in the olfactory bulb of the same four experimental groups two weeks post-lesion. Levels of synaptophysin in the lesioned and estrogen replaced group were significantly higher than in any of the other experimental groups.



Discussion

Previous studies have shown that estrogen loss is a major risk factor for AD. Significantly more AD patients are females than males, owing to the loss of estrogen during menopause. Furthermore, recent studies have shown that estrogen replacement therapy reduces the risk, delays the age of onset and slows the progression of AD (Paganini-Hill and Henderson, 1996). Estrogen loss is not an issue for males, because they produce endogenous estrogen throughout their lives, by converting testosterone into estrogen in adipose tissue through the enzyme aromatase (Hadley, 1996). How estrogen is involved in nerve repair is unclear and this study has shed light on this complex process.

In order to test the effects of estrogen on apoE levels and nerve regeneration, the olfactory nerve of mice was used as a model. The olfactory system provides a convenient model for this study. This model is well suited to nerve regeneration studies since the olfactory receptor neurons are able to regenerate. Also, the olfactory bulbs are the only system in the CNS in which total degeneration can be experimentally induced. These two properties make the olfactory bulbs an ideal model for this study.

Studies on the effects of lesioning and estrogen on apoE levels in the olfactory bulb revealed that estrogen is required for upregulation of apoE levels. The levels of apoE in the TX+E2 group were significantly higher than those in the TX+PL group. This finding is consistent with a previous finding that showed a 1.5-fold increase in apoE levels in the olfactory bulb following olfactory nerve lesioning in mice (Nisar, 1999). In contrast to the lesioned group, the non-lesioned groups (SL+E2 and SL+PL) showed no increase in apoE levels. The latter finding demonstrates that injury is necessary for

upregulation of apoE expression. Estrogen, in the absence of injury, had no significant difference from placebo-treated group. These data suggest that estrogen increases apoE levels in the olfactory bulb only when the olfactory nerve is injured.

Glial fibrillary acidic protein (GFAP) is a marker for astroglia (the cells which produce apoE in the brain). Olfactory nerve lesioning dramatically increased the GFAP levels in mice. This finding is consistent with previous findings (Poirier et al., 1991). Interestingly, GFAP level was not influenced by the availability of estrogen, since there is no significant difference in the GFAP expression between TX+E2 and TX+SL. Also, estrogen administration, in the absence of injury, had no significant effects on GFAP levels in the bulb. Mice from both SL+E2 and SL+PL had similar amounts of GFAP in the olfactory bulb. These data demonstrate that astroglia are either recruited or activated at the site of injury, presumably to provide apoE and other components to facilitate nerve repair.

Olfactory marker protein (OMP) was used as a marker to monitor actual nerve regeneration in the olfactory bulb. Two weeks following nasal irrigation of TX the OMP levels in the TX-E2 group was about 70% of normal levels (OMP levels in SL+PL). This data is consistent with previous studies (Nisar, 1999). Interestingly, the OMP level is lower in the TX-PL group as compared to the TX-E2 group. In the TX-E2 group, OMP levels are about 40% of the non-lesioned groups two weeks after lesioning. This indicates that regeneration is more complete in the estrogen replaced and lesioned group of animals, whereas, repair is delayed in the lesioned group without estrogen. Again, there is no difference in the non-lesioned groups where OMP level is presumably normal.

Synaptophysin is a protein expressed in the synaptic terminals, and therefore, is commonly used as a marker for synaptogenesis. The TX+E2 group showed higher levels of synaptophysin than did any other groups. Synpatophysin levels in the TX+PL group were significantly lower than any of the other groups. There was no significant difference between the non-lesioned groups. These levels of synaptophysin are presumably normal levels. It is not clear as to why synaptophysin levels in the TX+E2 are higher in the lesioned and estrogen replaced group than in the non-lesioned groups. It may be because neurons produce many axons and dendrites in the early stages after nerve repair. However, these projections and connections are pruned during the later stages of repair process, (Purves et al., 1997). In the absence of estrogen, the TX+PL group had decreased synaptophysin levels. This latter result is consistent with the decreased levels of OMP in the bulb.

Taken together these results suggest that the beneficial effects of estrogen on the nervous system are evident only in the presence of injury. These results are consistent with a recent study in humans. In this study, the effects of estrogen replacement therapy (ERT) on the cognitive and olfactory abilities of healthy post-menopausal women were investigated. It was hypothesized that women given ERT would show improvement in olfactory ability and cognition as compared to a placebo treated group. However, ERT had no detectable effects on either cognition or on olfaction. Parallel studies in mice subjected to long-term estrogen elevation found only transient changes in the synaptic density of olfactory neurons (Nathan, personal communication). These findings, along with the findings of this project, demonstrate that estrogen has no long-term effects on the CNS of neurologically intact subjects.

The data from this study also provides the pathway whereby estrogen facilitates nerve repair. In the presence of estrogen, nerve injury results in an increase in the number of astroglia at the site of damage. These glial cells produce and release apoE (Boyles et al., 1985; Pitas et al., 1987; Poirier et al., 1991). This increased production of apoE then facilitates nerve repair probably by redistributing lipids from the degenerating nerve terminals to regenerating axons of the olfactory nerve. Enhanced regeneration of olfactory receptor neurons then results in increased synaptogenesis as shown by the synaptophysin data.

In conclusion, estrogen has dramatic effects on apoE levels in an injured olfactory system. This pioneering study outlines the pathway whereby estrogen and apoE may work in concert to bring about nerve repair. More research is needed with regard to the interaction of estrogen and apoE and possibly other components and their functions in nerve repair. This line of research promises to shed light on many of the unknown disease processes at work in devastating neurodegenerative diseases, such as AD. Uncovering how apoE isoforms affect AD and what roles estrogen play in neuroprotection may very well lead to means of prevention and treatment of this devastating disease as well as other neurodegenerative diseases. It is very important that research at the cellular and molecular level of nerve damage in the CNS is persued.

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